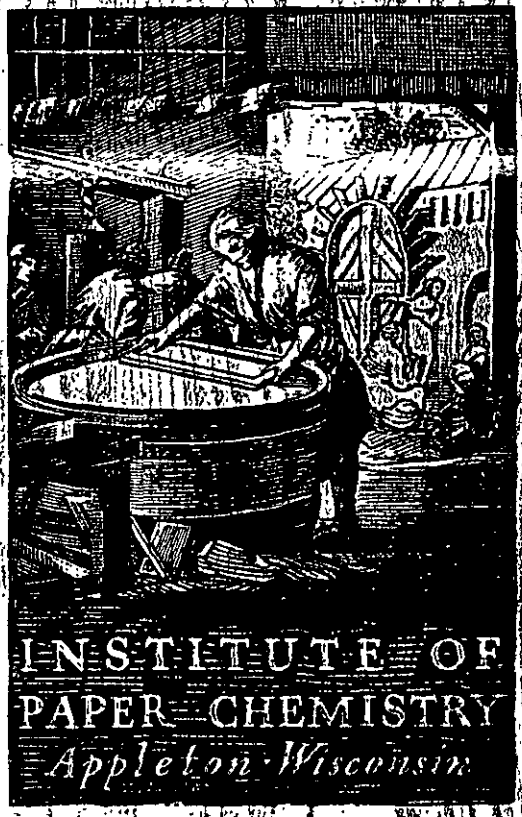

PARAMETERS OF ASPEN TISSUE CULTURE

Project 2351

Report Five

A Progress Report

to

PIONEERING RESEARCH COMMITTEE

July 28, 1965

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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SUMMARY

During the past ten months, the new investigator on this project conducted a series of familiarization and exploratory tests. Some causes of the variation in growth were determined, and indications were found of the nutritional requirements of aspen tissue.

Newly-isolated triploid quaking aspen tissue gave the same growth rate as tissue isolated three years ago.

A measure of variation in growth rate was obtained by individually weighing pieces of tissue at the beginning and end of each test.

Smaller pieces of inoculum, of about 3.1 mg. each, increased in weight by 65.7 mg. (1992%), while those weighing 8.3 mg. had a greater weight increase of 99.3 mg., but a lower per cent increase of 1065.

One piece of tissue per plate averaged 819 mg., compared to 457 mg. per plate when four pieces were used.

An agar concentration of 0.8% (the same normally used in Medium 23) was found to be best for general culture. However, early fast-growth might be obtained under some conditions using 0.5% agar.

Individual pieces of tissue grown in screw-top, one-ounce French-square bottles, showed their best growth when given some fresh air and light daily. Tissue disturbed daily, or in bottles kept closed in continuous dark gave less growth. Good growth was obtained using one and two pieces per bottle, but three pieces reduced total growth.

In the nutritional studies, water normally used from a Barnstead de-ionizer gave conductivity readings and tissue growth as good as distilled and double-distilled water. Tap water was unacceptable.

Freezing coconut milk for several months resulted in the loss of half of its growth promoting properties, when tested against fresh coconut milk in Medium 23.

Potassium iodide neither enhanced nor suppressed growth when added to Medium 23.

Iron supplied to Medium 23 in the form of Fe-EDTA, instead of Fe-citrate, gave faster but nonuniform growth of tissue during isolation and first subculture. However, one clonal line selected during the second transfer shows good, apparently uniform growth after six subcultures. Tissues isolated from six species of Populus in 1961 are now being maintained on Medium 23 supplied with Fe-EDTA.

In the search for a chemically-defined medium, variants of Wolter's media were tested in both agar and liquid forms. Essentially, these media replaced coconut milk with myo-inositol, used Fe-EDTA, replaced NAA with 2,4-D and kinetin, and doubled the salts used in Medium 23. Wolter's two media were developed for diploid quaking aspen tissue. Our early studies indicated that for triploid tissue, myo-inositol apparently replaced coconut milk when supplied in the presence of Fe-EDTA and NAA. However, later studies showed that something had been carried over from the coconut milk and used in the first transfer, but was soon exhausted in the second transfer.

In determining which vitamins in addition to inositol might replace coconut milk, the first approach was to add one vitamin at a time to the basic

defined Medium 32. This basic medium contained the vitamins inositol and thiamine, as well as the auxin NAA. Several individual vitamins appeared promising; but results varied between isolation and the first and second transfers, as well as between agar and liquid cultures.

Other studies included an exploration of pollen and single cell cultures, the isolation of several promising strains of aspen tissue, and the discovery of one piece of differentiated tissue having three roots and two elongating shoots.

INTRODUCTION

The three-year grant for Project 2351 terminated April 30, 1965. Progress Reports One through Four were submitted to the Pioneering Research Committee by Dr. Martin C. Mathes, the principal investigator. These reports covered his work from the initiation of the program until August 30, 1964, when Dr. Mathes was succeeded by Dr. Lawson L. Winton.

This report describes the work of Dr. Winton from September 1, 1964, to June 30, 1965, and is the final report on the original three-year project. However, this program is being continued, and a proposal has been submitted requesting an additional three-year grant by the Pioneering Research Committee.

In Progress Report One, Mathes described the successful isolation and growth of undifferentiation callus tissue from aspen trees. A suitable nutrient Medium Number 23 was developed, consisting of major and minor salts, sucrose, coconut milk (CM), naphthateneacetic acid (NAA), glycine, thiamine, and 0.8% agar. Attempts to develop a chemically defined medium were unsuccessful.

Tissue was isolated in late 1961 from Populus davidiana, P. alba, P. grandidentata, P. canescens, P. deltoides (eastern cottonwood), and P. tremuloides (quaking aspen). Tissue was isolated from both diploid and triploid trees of quaking aspen; however, tissue from the former failed to grow on Medium 23. Cultures have been maintained continuously for all species, but emphasis has been placed on triploid quaking aspen – a tree of possible future major importance to the paper industry.

The long-range goals of Mathes were to develop tissue culture as a tool to study the physiology of aspen, as well as to eventually obtain independent plants from differentiated callus tissue.

In an effort to control the differentiation of callus tissue, Mathes was able to significantly increase the production of roots by the addition of 0.05% citric acid to Medium 23. Unfortunately, shoot initiation was not consistent and shoot elongation did not occur.

Studies were conducted on antimicrobial substances which were found to diffuse from callus tissue into the nutrient medium. The chemical compositions of intact, as well as isolated, callus tissue were determined. Also a high correlation was shown to exist between the rate of tree growth and the rate of callus production.

During the past several months, emphasis has been returned to the original objectives of this program, and a long-range plan submitted which will explore the techniques, nutrition, and environment required for the differentiation of callus tissue. This plan will include (1) the measurement and reduction of variation in tissue growth, (2) the development of a chemically defined medium, (3) the establishment of populations of single cells in liquid suspensions, and (4) the control of differentiation from single cell to mature plant.

VARIATION STUDIES

OLD VS. NEW TISSUE

The growth rate of triploid quaking aspen callus tissue, isolated in 1961 (1), was compared to that of the same type of callus isolated only 21 days previous to this test. Five pieces of tissue from the T-2-56 triploid aspen clone were placed on Medium 23, in each of five plates, on November 30, 1964. After 22 days incubation in the dark at 28°C. the tissue was weighed and the results analyzed.

The average fresh-weight increase of newly isolated tissue was 179 mg. per piece, compared to 170 mg. for tissue from the long-term cultures. The respective per cent increases in weight were 1301 and 1164, and were not different at the 5% level of significance.

These results indicate that as long as only the white, rapidly growing tissue on callus pieces are used, regardless of the number of past subcultures, no difference in growth may be expected between old and new tissue isolates. Further data on this subject is given later under the Fe-EDTA studies.

VARIABILITY OF CALLUS GROWTH

Mathes (1) found considerable variation in the rate of growth of triploid aspen tissue, and, therefore, performed all experiments at least three times. In most studies, 5-10 pieces of tissue were placed on Medium 23 in each of five Petri dishes per treatment. Usually all data were pooled and reported for each experiment as a single per cent increase in growth for each treatment. Statistical analyses of the results were thus hindered. This mass approach was imposed, in part, by the time and difficulty of weighing pieces individually

under sterile conditions on a triple-beam analytical balance then in use. Fortunately, the recent purchase of a direct-weigh balance has now made possible the rapid and sterile weighing of individual callus pieces.

A knowledge of the degree of variation of the rate of tissue growth would permit the selection of the minimum number of pieces and replications necessary which would show significant differences between treatments. The objectives of this study were to measure and determine the causes, and reduce the variations in the rate of growth of callus tissue.

Roots were collected from one tree of the triploid quaking aspen clone, T-2-56, on October 20, 1964. Following the method of Mathes (1), root sprouts 8-12 cm. tall (Fig. 1) were harvested several times in November and sterilized segments placed, bases out, on coconut-milk Medium 23 for the initiation of callus tissue. Early cultures were contaminated by an unidentified, but apparently systemic, gram-positive bacteria (Fig. 2). Contamination was reduced in later collections by discarding the lower 1/3-1/2 (lower internode) of each sprout, and increasing the time of surface sterilization in 5% NaOCl from five to eight minutes. The white callus tissue, which formed best on the basal ends of the 1-cm.-long segments, was then subdivided and used for the following variation studies.

Test A. On December 15, 1964, triploid aspen tissue, isolated 33 days previously, was subdivided and thirty pieces were individually weighed. Five pieces were placed on Medium 23 in each of six Petri dishes, which were then kept in the dark at 28°C. for 27 days. The average initial weight of pieces 1-10 was 11.3 mg., 15.6 mg. for pieces 11-20, and 9.3 mg. for 21-30. The average weight of the third group was significantly less than groups one and two. The average of tissue in all groups was 12.1 $\pm$ 1.5 mg. per piece (Table I).

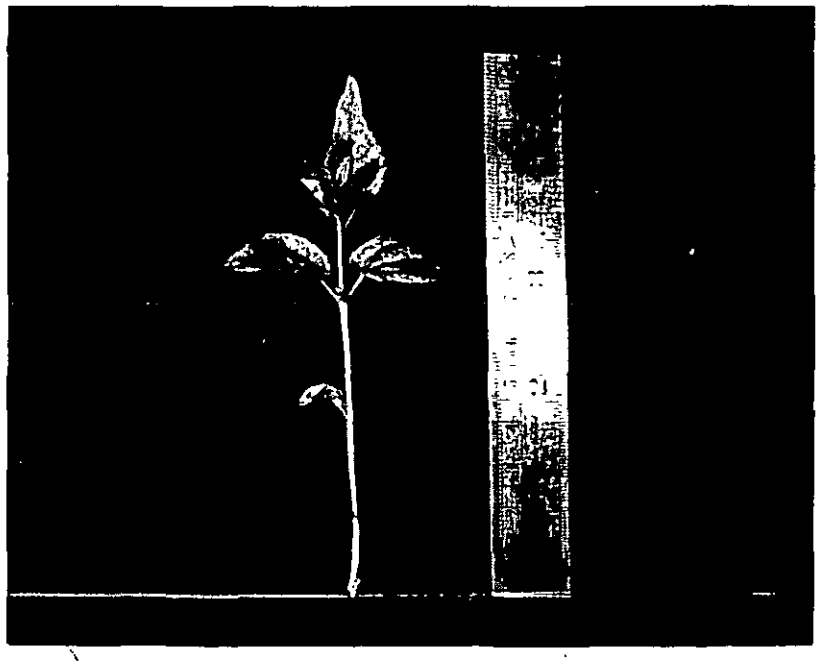

Figure 1. Root Sprout 9.5 cm. Tall, From the Triploid Quaking Aspen Clone T-2-56

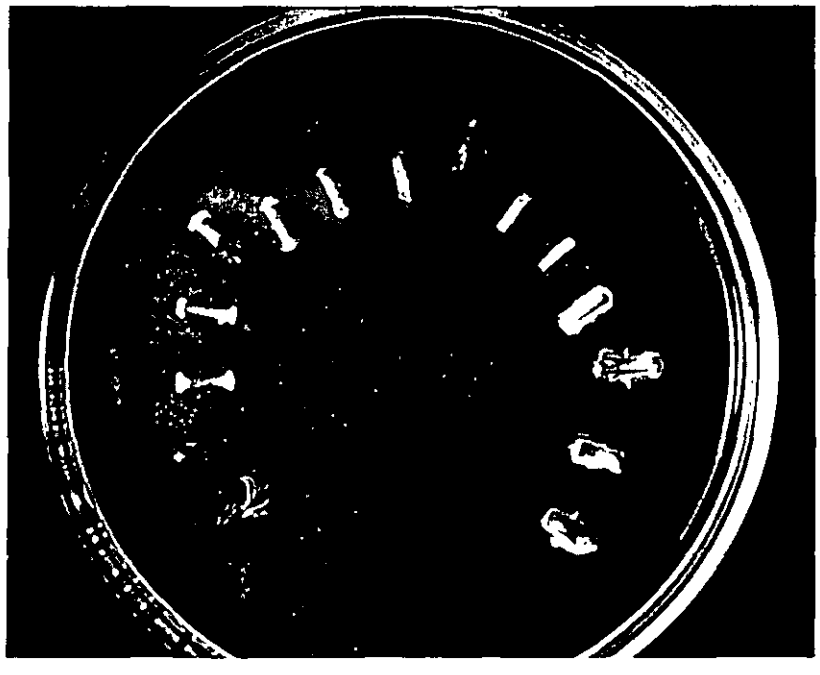

Figure 2. Segments of Root Sprout Arranged with Bases Outward. Callus Tissue Generally Grew Better on Basal Ends. Lower Segments Were Sometimes Contaminated by a Gram-Positive, Systemic Bacteria Seen on the Photograph as a Thick, White, Opaque Substance (Arrow)

TABLE I

AVERAGE INITIAL WEIGHT IN MG., AND FRESH WEIGHT INCREASE OF PIECES OF TRIPLOID QUAKING ASPEN IN TWO TESTS OF VARIATION

Pieces	Test A	Test B
	Initial Weight, mg.	
1-10	11.3	67.0
11-20	15.6	68.3
21-30	9.3	53.0
Total	12.1 ± 1.5	62.7 ± 8.7
Range	10.6 - 13.6	54.0 - 71.4
	Weight Increase, mg.	
1-10	270.8 ± 21.3	169.8 ± 22.4
11-20	270.8 ± 21.9	169.8 ± 24.3
21-30	270.8 ± 22.9	169.8 ± 23.9
Total	270.8 ± 22.0	169.8 ± 23.0
Range	248.8 - 292.8	146.8 - 192.8

To test the variation in the increase in fresh weight, callus pieces were analyzed by three different designs: (1) 10 pieces per treatment for 3 treatments, (2) 5 pieces per treatment for 6 treatments, and (3) 2 pieces per treatment for 14 treatments. There were no significant differences in the average weight increase of pieces in any of the three analyses. The average increase of all tissue was 270.8 ± 22.0 mg. per piece (Table I).

Test B. In the second test, 30 pieces of triploid aspen callus tissue, from the same source described above, were individually weighed on December 16, 1964. Ten pieces were placed on Medium 23 in each of three Petri dishes and incubated 25 days in the dark. The average initial weights in each dish were

67.0, 63.3, and 53.0 mg. per piece, and were not significantly different. The average of all tissue was 62.7 ± 8.7 mg. per piece (Table I). The average increase in weight of pieces tested 2, 5, and 10 pieces per treatment were not significantly different.

These results show that pieces of tissue which had different initial weights were not significantly different after 25-27 days' growth. On the basis of these two tests, pieces of all isolated callus tissue apparently grow quite uniformly on Medium 23. This may indicate that the variation in growth observed by Mathes perhaps was not statistically significant at the 5% level. The implication of this hypothesis is that without analyses of variance of individual-weight data, it would be impossible to tell whether fairly close per cent increases in weight reported from mass-weighing data represented real or apparent differences between treatments. By using individual weights, it may be that a high sensitivity to differences between treatments can be maintained by using fewer pieces and replications than were used in Tests A and B. This would mean a saving in both time and labor.

Studies on the variability of growth are being continued, especially on the relationship of callus tissue isolated from root sprouts raised at different times of the year.

INITIAL WEIGHT OF TISSUE

White and Risser (2) found that the rate of growth of spruce callus decreased with the increase in the size of inoculum used. Over a two-week period, they found that pieces weighing 5-10 mg. increased 920% while those of 31-35 mg. increased less than half as much, or 370%. This same general relationship was also observed for triploid quaking aspen tissue in the following test.

One large, white piece of newly initiated callus tissue was subdivided into eight pieces. The ninth piece was cut from a different piece of callus of the same parental material. One piece each of the tissue was placed on a 9 ml. agar slant of Medium 23 in a one ounce French-square bottle. All bottles were kept in the dark at 26°C. for one month (Fig. 3).

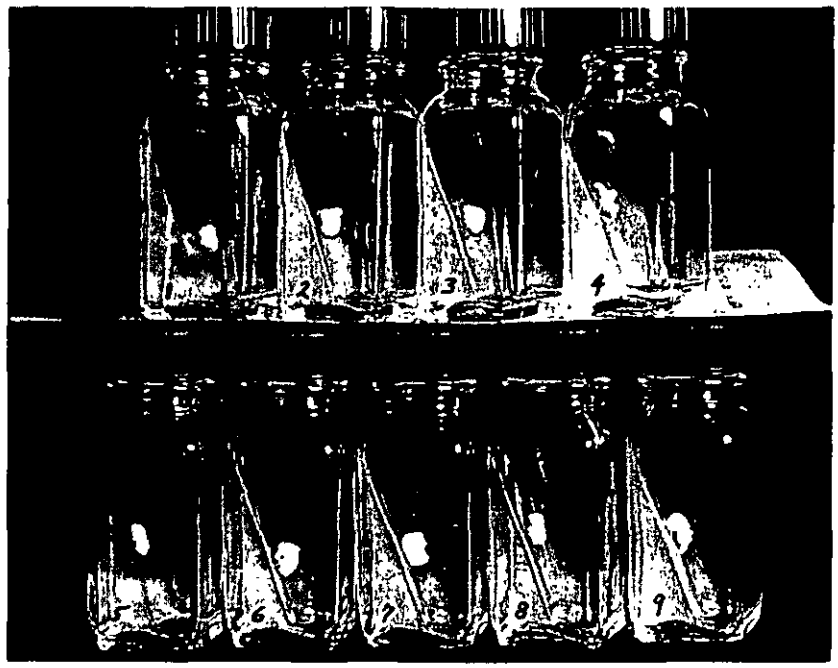

Figure 3. Clonal Pieces of Triploid Quaking Aspen Tissue, Grown in Individual French-Square Bottles on 9 ml. Slants of Medium 23

Table II shows that tissue averaging 3.1 mg. per piece increased 1992%, while those averaging 8.3 mg. (or almost 2.7 times as heavy) only increased 1065%. On the other hand, the total weight increase of the smaller pieces was 65.7 mg. compared to 99.3 mg. for the pieces having a heavier initial weight.

TABLE II

GROWTH OF PIECES OF CLONAL, TRIPLOID QUAKING ASPEN TISSUE
OF TWO DIFFERENT AVERAGE INITIAL WEIGHTS IN MG.

Treatment	Piece	Initial Weight, mg., 4/29/65	Final Weight, mg., 5/28/65	Weight Increase, mg.	Per Cent Increase
1	1	3.5	77.3	73.8	2009
	2	3.1	59.2	56.1	1710
	3	2.4	56.0	53.6	2133
	4	2.4	80.0	77.6	3133
	5	4.3	71.7	67.4	1467
	Av.	3.1		65.7	1992
2	6	9.2	93.3	84.1	814
	7	8.4	102.9	94.5	1025
	8	8.1	140.7	132.6	1537
	9	8.4	94.9	86.0	924
	Av.	8.3		99.3	1065

This experiment illustrates a possible danger of relating growth rates between treatments only in terms of per cent increase, especially when pieces of different initial weights are used in the same experiment. The smaller pieces clearly increased in per cent proportionally greater than the larger pieces. However, the total increase in weight of the larger pieces was 1.5 as great as for the smaller pieces. Since tissue quality is as important as tissue growth, further tests will be made with inocula of different sizes to determine the optimum initial size producing the best tissue. The optimum duration of growth will also be studied.

Despite the fact that the general relationship found by Risser and White was demonstrated here, an analysis of variance at the 5% level showed that

the weight increases were not significantly different among tissue grown from pieces having different initial weights.

NUMBER OF PIECES PER PLATE

From a clonal strain of triploid quaking aspen tissue (3x-B), pieces of fresh material (ranging from 40 to 137 mg. each) were placed on Medium 23E (Fe as Fe-EDTA instead of Fe-citrate). One piece was placed in each of two Petri dishes, as well as in each of two 250 ml. Erlenmeyer flasks on April 20, 1965. In addition, four pieces were placed in each of two Petri dishes. Individual initial and final weights were recorded.

After 38 days' incubation in the dark at 26°C., the average increase in weight for one piece per plate was 819 $\pm$ 140 mg. and for four pieces per plate 457 $\pm$ 120 mg. (Fig. 4). The differences were highly significant.

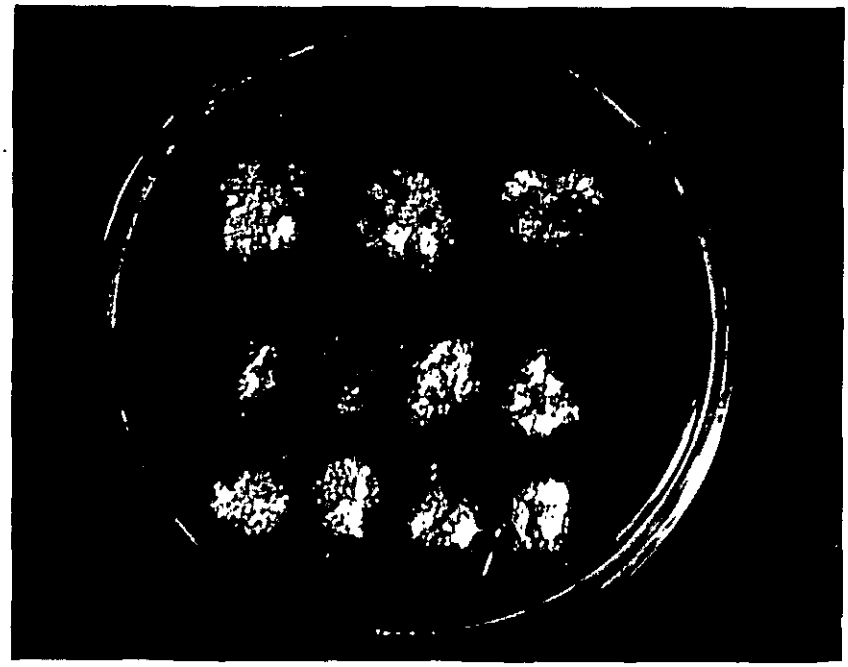

Figure 4. Aspen Tissue in the Top Row Grew From Single Pieces Per Plate. Each of the Two Sets of Four Pieces in the Middle and Bottom Rows Grew on Separate Plates. The Difference in Weight Increase Between One and Four Pieces Per Plate Was Significant

AGAR CONCENTRATION

Mathes originally used 0.9% Difco-Bacto agar in his coconut milk Medium 23, which was later reduced to 0.8%. White and Risser (2) found that 0.5% Noble agar gave satisfactory growth for their spruce tissue. The purpose of this study was to determine whether the rate of growth and quality of aspen tissue is influenced by the concentration of agar in the nutrient medium.

On April 2, 1965, four pieces of tissue per plate, and seven plates per four concentrations, were prepared using 0.80, 0.85, 0.90, and 0.95% Bacto agar. The estimated initial weight per piece was 3 mg. After 18 days' growth, all pieces per plate were weighed together, and the average increase in fresh weight per piece was found to be 182, 178, 172, and 158 mg., respectively, for the four agar concentrations. These weights were not significantly different. However, the best growth was obtained on 0.8% agar, which also had a slightly moist, spongy surface. As concentration increased, the surface became slightly drier and firmer.

In a second test, three pieces of tissue, weighing about 5 mg. apiece, were placed in each of three plates for each treatment of 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0% Bacto agar. The most rapid growth occurred on 0.5% agar (324 $\pm$ 182 mg.), compared to 214 $\pm$ 64 mg. and 203 $\pm$ 86 mg. for 0.8 and 0.9% agar, respectively. However, mass weights instead of individual weights were used, probably causing the wide overlapping ranges of average weights, resulting in insignificant differences.

This study will be continued, using pieces of tissue of uniform initial weight. One advantage of using the relatively drier 0.8% instead of the wetter 0.5% agar, would be the reduction of the spread of occasional fungus contaminations.

On the other hand, more rapid growth may be obtained for special studies by using 0.5% agar during the first few weeks of growth. Pieces of tissue over 1 cm. in diameter tended to become necrotic when grown in the more fluid 0.5% agar, in contrast to a healthier yellow color on 0.8% agar.

GROWTH IN CLOSED BOTTLES

White and Risser (2) preferred the use of one ounce French-square bottles, having screw caps, in which to grow spruce callus tissue. Theirs is the latest report of using closed bottles, which are a departure from the rather standard methods of using open vessels loosely closed with cotton (flasks and test tubes) or glass (Petri dishes).

In our work, Petri dishes are normally used but do become contaminated occasionally. The loss of plates by contamination forces the use of sophisticated analytical tests, which might possibly be eliminated by switching to a closed-vessel culture system.

The purposes of this study were to determine whether aspen tissue will grow better (1) in closed bottles rather than in Petri dishes, (2) when one rather than two or more pieces of tissue are used, and (3) in constant darkness rather than in intermittent or subdued light. In addition, a growth curve was plotted, and the effects tested of removing, weighing, and replacing tissue from its nutrient agar daily.

Newly isolated tissue from triploid quaking aspen was subdivided on April 14, 1965, and used as inocula weighing 4-5 mg. per piece. Sixty one-ounce French-square bottles were used, each containing 9 ml. of Medium 23 on a slant. Each of the five treatments shown in Table III was tested in a separate set of

TABLE III

THE TREATMENT, NUMBER OF PIECES, AND WEIGHT IN MG. OF TRIPLOID ASPEN TISSUE GROWN IN BOTTLES AND PETRI DISHES ON MEDIUM 23

Set	Vessel	Treatment[a]	Vessels Used	Pieces/ Vessel	No. Pieces Analyzed Per Set	Total Weight Per Set, mg.	Av. Weight Per Piece, mg.
A	Bottles	a	10	1	7	886	126
B		b	10	1	10	2129	213
C		c	10	1	10	1467	147
D		d	10	1	10	986	99
E		d	10	2	20	1922	96
F		d	10	3	27	1688	62
H	Petri dishes	e	5	1	5	1162	232
I		e	5	2	8	1966	246
J		e	5	3	15	3825	255
K		e	5	4	20	5127	256
L		e	4	5	27	5280	196

[a] Treatments:
a. Opened and pieces removed – in the light daily.
b. Opened and pieces intact – in the light daily.
c. Sealed and pieces intact – in the light daily.
d. Sealed – in constant darkness.
e. Tops loose – in constant darkness.

ten bottles. One piece of tissue, weighing 28-87 mg., was placed in each bottle of Sets A through D. Tissue received different combinations of disturbance (weighing), light, and air. Each piece per bottle in Set A was removed and weighed daily. Bottles in Set B were opened daily but pieces left intact. Bottles in Set C were brought into the light daily with Sets A and B but remained unopened. Sets D, E, and F remained wrapped in foil in the incubator, and contained one, two, or three pieces of tissue, respectively, per bottle.

The results of this rather complicated study were not analyzed statistically. However, several important relationships were observed which will be the basis for further tests of one factor at a time. These are as follows:

1. The growth rates, determined only for pieces in Set A which were disturbed daily, showed fairly uniform increases in fresh weight per piece. However, the rates varied between pieces from an initial 3-5 mg. to pieces with weight increases ranging from 70 to 130 mg.

The best growth was obtained from piece number one of Set A (Fig. 5A and 6A), which had the slowest start but finally attained the greatest weight of 267 mg. This was more than twice the weight of any other piece in Set A. The agar in six bottles of this set became contaminated during the experiment, but only three pieces of tissue had to be discarded.

In addition to occasional contamination, opening the bottles daily and removing each piece of Set A appeared to reduce growth by 40% from that recorded for Set B. Tissue A also changed from hard light-yellow to a darker, spongy texture. Pieces in Set B (operated daily) were left intact. Bottles in Set C were left sealed, although brought into the light for ten minutes daily with Sets A and B, but only had 30% of the growth recorded for Set B. Pieces sealed in

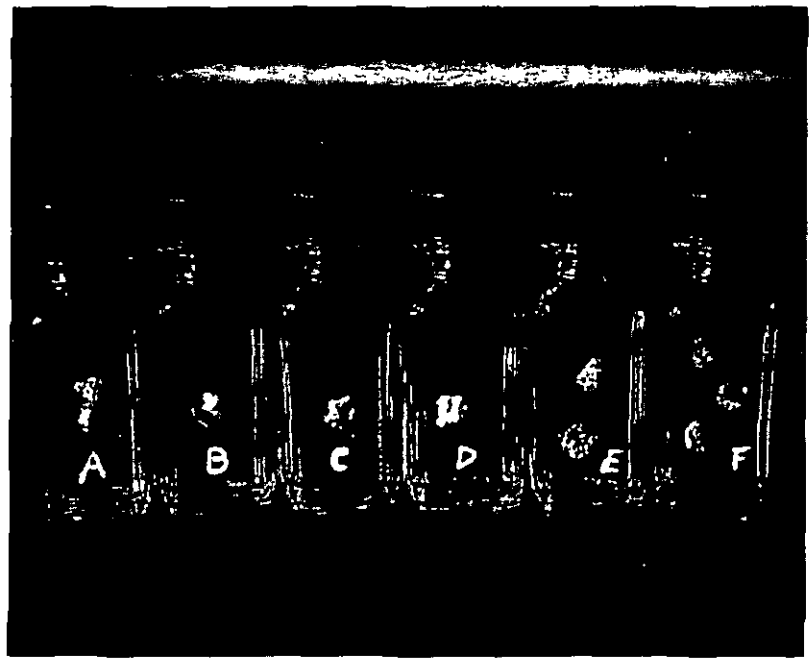

Figure 5. Intact Pieces Are Shown of Triploid Quaking Aspen Tissue in One Bottle From Each of Sets A, B, C, D, E, and F, From Left to Right. See Table III for an Explanation of Treatments

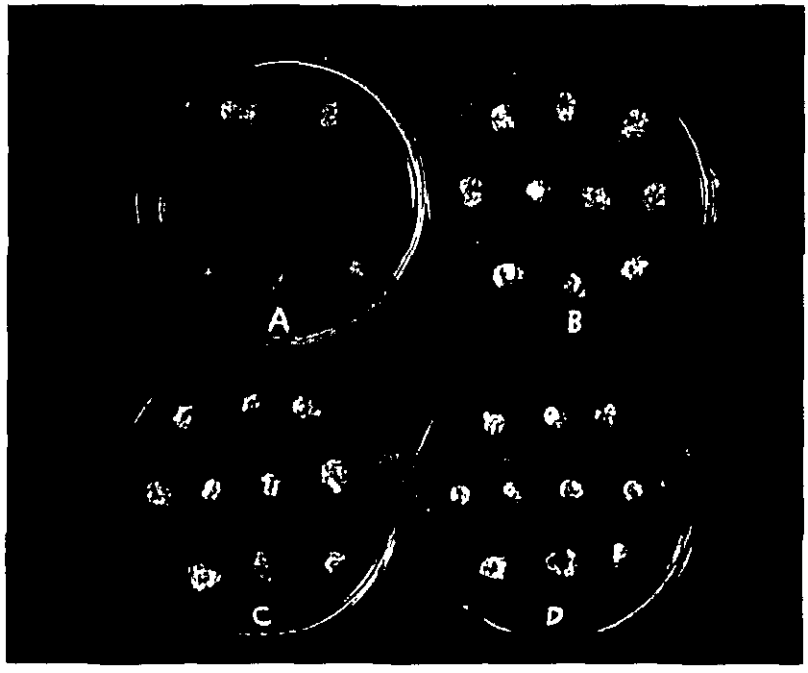

Figure 6. Pieces of Tissue Arranged on Plates Which Were Individually Grown in Bottles. Piece Number One of Each Set Is in the Upper Left. See Table III for an Explanation of Treatments

bottles of Set D and left in constant darkness had 53% less growth than Set B (see Fig. 5 and 6 and Table III).

These results indicate that, when using one piece of tissue per bottle, fresh air and occasional light give better growth than no air, with either occasional or no light. Reducing the duration of growth from four to two weeks may reduce the need for fresh air.

2. In another comparison, bottles containing either two or three pieces of tissue were left in total darkness. Tissue shown in Fig. 7 is arranged on plates for easier examination. An apparent decrease in size is evident in Sets D, E, and F as 1, 2, or 3 pieces of tissue were respectively grown in one bottle. However, because of the large variation between pieces, the average weight of pieces in Set E was only slightly less than Set D (Table III), and pieces in Set F averaged about 41% lighter than those in Set D.

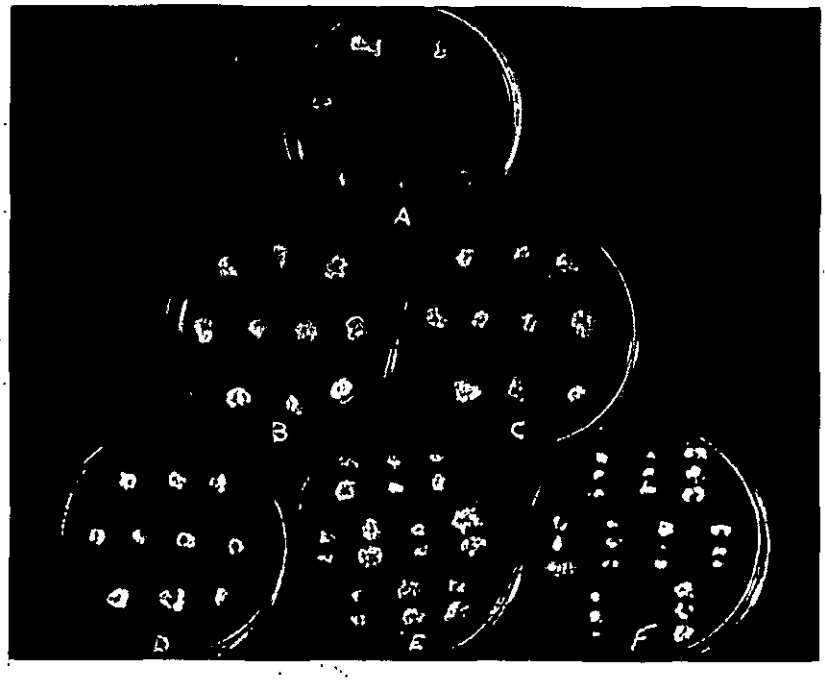

Figure 7. Pieces of Bottle-Grown Tissue Are Arranged on Plates According to Treatments A-F. In Dishes E and F Are Shown Groups of Two and Three Pieces, Respectively, Grown in Single Bottles

Clearly, three pieces of tissue per bottle inhibits growth, but apparently two pieces could provide twice the total tissue per experiment as obtained from one piece per bottle.

3. Using Petri dishes, the average increase in weight of tissue was about the same (232, 246, 255, and 256 mg. per piece), whether 1, 2, 3, or 4 pieces, respectively, were grown on one plate (Fig. 8 and 9). Only when nine pieces were grown per plate did the average weight fall to 196 mg. (Table III). On the basis of efficiency, about two and a half times the total amount of tissue was grown per Petri dish containing four pieces of tissue on 33 ml. of medium, as in one bottle containing one piece on 9 ml. of medium. On the other hand, using two pieces per bottle of high-quality noncontaminated tissue may prove to be the better method, especially when using clonal material of low growth variability.

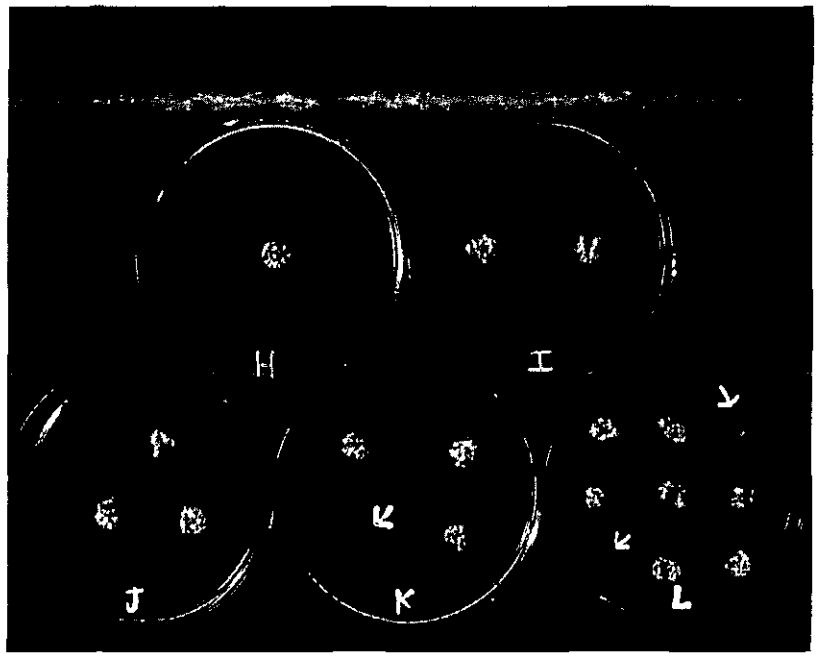

Figure 8. One Original Plate of Each Set Having 1, 2, 3, 4, or 9 Pieces Per Petri Dish. Note Spontaneous Friable Tissue (Arrows)

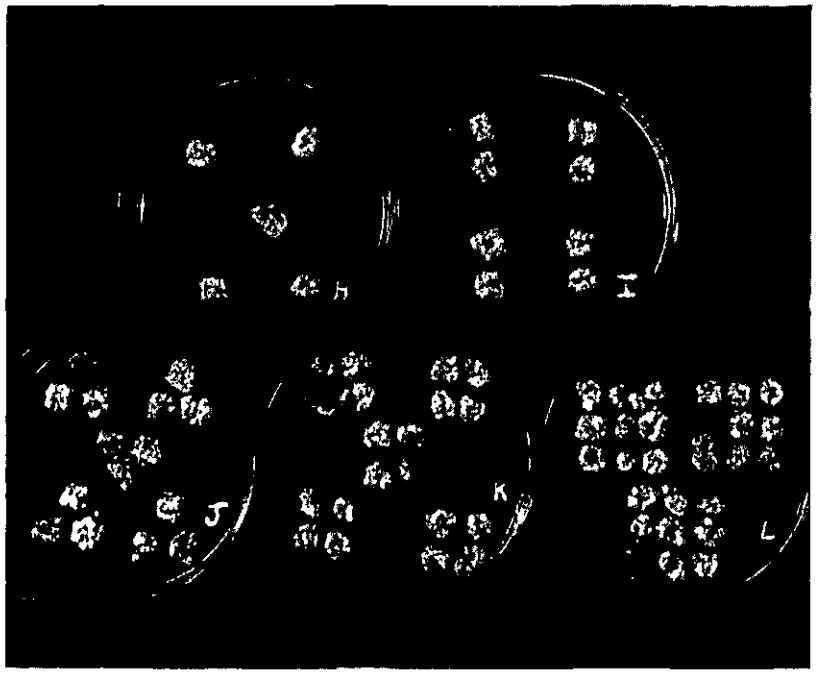

Figure 9. All Pieces of Sets H-L Placed on One Plate Per Treatment for Easier Observation. Pieces Were Grown 1, 2, 3, 4, or 9 Pieces Per Petri Dish

NUTRITIONAL STUDIES

WATER ANALYSIS

For several years, this laboratory has used a Barnstead deionizer (blue cartridge) as a water source for the preparation of Medium 23. This water was tested against samples from three other sources to determine whether optimum growth was being obtained in tissue cultures.

On December 7, 1964, samples of double-distilled, distilled, deionized, and tap water were collected and stored in pyrex flasks and polyethylene bottles. Two days later, 200 ml. of each sample were used to make Medium 23. Five pieces of callus tissue, averaging about 60 mg. apiece, were placed in each of five plates per treatment. The tissue was incubated for 26 days in the dark at 28°C. On December 12, conductivity tests were run on the remaining 200 ml. of each sample.

Table IV gives the conductivity of each water sample in micromoles per centimeter squared, equivalent to a potassium chloride standard. Also given are the respective average values of the fresh-weight increase, as well as the per cent increase of tissue grown on media made with each water source. Analyses of variance were performed, both including and excluding data from Sample 6 (tap water). These results showed that growth on Medium 6 was much less than on the other five media (Fig. 10), which had no significant differences in growth among them.

Both the distilled and double-distilled water stored in pyrex gave lower conductivity readings than those stored in polyethylene. On the other hand, growth of tissue on media made with water stored in polyethylene was

slightly higher than for water stored in pyrex. This was true as a per cent increase as well as a net increase.

TABLE IV

CONDUCTIVITY OF WATER SAMPLES AND GROWTH OF TRIPLOID QUAKING ASPEN TISSUE ON MEDIA MADE WITH EACH WATER SAMPLE

Water Source	Container	Conductivity in micromoles/sq.cm.	Increase per Treatment Net	Increase per Treatment Per Cent
1. Double distilled	Poly	2.18	178	1321
2. Double distilled	Pyrex	1.58	213	1616
3. Distilled	Poly	5.69	206	1400
4. Distilled	Pyrex	1.66	206	1530
5. Deionized	Poly	4.60	224	1662
6. Tap	Poly	242	136	1003

According to this study, Medium 23 made with deionized water produces as good growth as when made with distilled or double-distilled water. Tap water gave significantly less growth than any of the others. Apparently, the plasticizers in polyethylene contribute an energy source; but since our medium is not normally made in polyethylene containers, this point will not be pursued. Our present source of water appears to be of sufficient quality so as not to be a major factor in the variation of tissue growth.

FROZEN COCONUT MILK

Coconuts are available throughout the year. However, observations in this laboratory indicate that the quality of the milk may vary somewhat throughout the year. This test was performed to determine whether coconut milk can be stored by freezing, and what effects this might have on the growth of triploid aspen tissue.

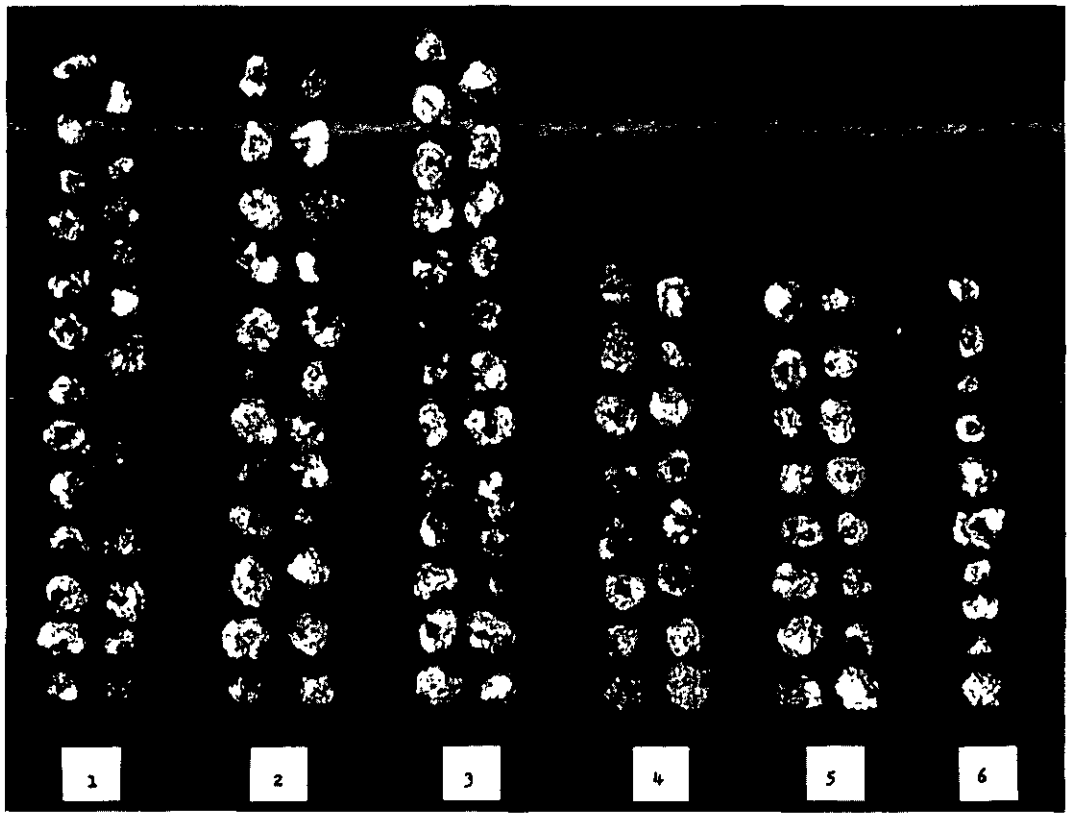

Figure 10. Tissue Grown on Media Made With Different Water Samples. Some Tissue Was Lost from Treatments 4, 5, and 6 by the Drying of Agar from the Plates. The Only Significantly Poor Results Were on Medium 23 Made With Tap Water (6)

On November 20, 1964, 100 ml. of fresh coconut milk were placed in a polyethylene bottle at -10°C. The milk was thawed March 15, 1965, and, though cloudy, was used to make one liter of Medium 23. The fresh coconut milk used in the control Medium 23 was clear after heating and filtering. Four pieces of tissue, each weighing an estimated 40 mg., were placed in each of six plates of Medium 23 made with fresh and with frozen coconut milk.

The average increase in fresh weight per piece was 116 mg. using fresh, and 57 mg. using frozen coconut milk. This difference was significant, showing that freezing the coconut milk apparently causes the loss of about half of its growth promoting properties.

KI IN MEDIUM 23

The major elements suggested by White (3) are used in this laboratory for the formulation of Medium 23 (1). However, in reviewing the laboratory instructions for the preparation of this medium, it was noted that potassium iodide had not been included with the major salts as specified by White. Thus, a test was run to determine whether KI enhances the growth of triploid aspen tissue.

On November 11, 1964, five pieces of aspen tissue, averaging 11-15 mg. each, were placed in five Petri dishes for each of the two treatments of 23 medium and 23 plus 0.5 mg./l. KI. After 41 days, pieces grown on Medium 23 averaged 352 mg. and those on 23 plus KI 314 mg. Differences were not significant. The addition of KI did not appear to either inhibit or enhance growth.

Fe-EDTA AND CLONAL LINES

Klein and Manos (4) and Wolter (5) suggested using iron in the nutrient solution which had been chelated with disodium ethylenedinitrilotetraacetate (EDTA). Wolter found that 5.6 mg./l. of Fe as Fe-EDTA gave satisfactory growth of diploid quaking aspen tissue. The following series of experiments was designed to test whether tissue from the triploid form of quaking aspen would benefit from Fe-EDTA more than from Fe-citrate normally used in Medium 23.

Test A - Isolation

Three replications of two plates each were prepared January 16, 1965. One plate of each replicate contained Medium 23 and the other 23E (Fe-EDTA). Four pieces of newly-isolated triploid aspen tissue and four pieces of tissue cultured since 1961 were placed on respective halves of each of the six plates. Pieces were weighed individually at the start of the study, and ranged from 7

to 18 mg. each. Tissue was incubated in the dark for 28 days, then all pieces were again weighed individually.

A summary of the weight increases of old and new tissue on Medium 23, as well as old and new tissue on 23E is given in Table V.

TABLE V

INCREASE IN WEIGHT IN MG. OF OLD AND NEW TRIPLOID QUAKING ASPEN TISSUE IN TEST A, AFTER 28 DAYS' GROWTH ON MEDIUM 23 OR 23E

Treatment		Total Weight of Four Pieces Per Plate, mg.			Average Weight
Medium	Tissue	Rep. 1	Rep. 2	Rep. 3	Per Piece, mg.
23	Old	971	986	1281	270
23	New	878	1080	1289	271
23E	Old	1506	982	1081	297
23E	New	1802	875	1104	315
Total		5157	3923	4755	

No significant differences were found between treatments in the two-way analysis of variance. However, differences were observed between replications, as well as for the interaction (experimental error) between treatments and replications, i.e., variation was greater between pieces receiving the same treatment than between pieces receiving different treatments. A closer examination of the data indicates that newly isolated triploid aspen tissue, grown on 23E, apparently is too variable to use as an experimental material. Occasional pieces of tissue grown on 23E were larger than the average piece grown on Medium 23. However, fresh tissue was generally whiter and pieces more uniform in size when grown on Medium 23 rather than 23E.

Test B – First Subculture

On February 12, 1965, each of callus pieces, numbers 1 through 32, in Test B, was completely subcultured. All of each callus was transferred to separate plates containing the same 23 or 23E medium used in the first test. Three to ten pieces of white tissue were placed in the center and the remaining 10-20 pieces were placed around the edge of the plate.

After 21 days, the total weight of tissue in each plate was recorded These data are summarized in Table VI, which also gives the increase in growth for each treatment relative to the best growth of the test, i.e., new tissue on 23E. As in Test A, there were no differences between treatments, but differences between replications and interaction were significant. Thus, variations of growth of new tissue on 23E were still greater than between treatments.

TABLE VI

INCREASE IN WEIGHT IN MG. OF OLD AND NEW TRIPLOID QUAKING ASPEN TISSUE IN TEST B, AFTER 21 DAYS' GROWTH ON MEDIUM 23 AND 23E

Treatment		Total Increase in Weight of Tissue on Four Plates Per Treatment, mg.		Average Increase	Per Cent Growth
Medium	Tissue	Rep. 1	Rep. 2	Per Plate, mg.	of 23E – New
23	Old	8770	8940	2214	79
23	New	8870	8647	2190	78
23E	Old	11239	9462	2588	92
23E	New	13498	8881	2797	100
Total		42377	35930		

Test C – Eleven Weeks' Growth

The remaining tissue (Pieces 33-48) in Test A were transferred to new medium on March 5, 1965, but were not subcultured as were pieces in Test B. The four pieces per plate in Test C represented one treatment each of Test A.

The total increase in weight per treatment, during the 11 week period, was 3718 mg. for old tissue on Medium 23, 4219 mg. for new tissue on 23, 4580 mg. for old on 23E, and 4926 mg. for new on 23E. The differences are not significant. Each piece was about one centimeter in diameter and half that in height, consisting mainly of dark-brown callus having some white tissue on top.

Test D – Second Transfer and Establishment of Clonal Lines

On March 5, 1965, the single best piece of tissue was selected from three of the four treatments, at the conclusion of Test B. The white tissue on each piece was cut into four new pieces (of about 10 mg. each), and placed on one plate containing the same medium used in Test A.

The increase in total weight per treatment was 731 mg. for old tissue on Medium 23, 1038 mg. for old tissue on 23E, and 1217 for new tissue on Medium 23E. The most uniform white tissue was on old tissue on 23E, which originally came from Plate 28 of Test B. Thus, a clonal line of 3x-28 on 23E was initiated and has been maintained to the sixth subculture as of July 7, 1965.

Test E – Clonal Lines of Populus Species

Late in 1961, Mathes (6) isolated tissue from Populus deltoides, P. davidiana, P. grandidentata, P. alba, and P. canescens, in addition to both diploid and triploid P. tremuloides. Cultures of all but the diploid quaking aspen have been maintained until recently on Medium 23, which furnishes iron as Fe-citrate.

On February 16, 1965, 9 to 10 pieces were cut from each of two callus pieces per species and placed on one plate each of Medium 23 and 23E. After 24 days in the incubator, there was very little difference between media in the growth of tissue from most species. The best white growth for all species was on 23E, but variations were also greater.

A second test, identical to the one outlined above, also showed the best growth to be on 23E. An exception to this was the triploid quaking aspen, which had the best growth of white tissue, as well as higher uniformity on Medium 23.

After several subsequent subcultures and transfers on both media, only tissue from triploid quaking aspen was thereafter maintained on Medium 23. Tissue from the other five species has been maintained on 23E for 6 or 7 subcultures, with the result that exceptionally uniform material of high quality is constantly available for experimentation.

CHEMICALLY-DEFINED MEDIA

Wolter's Media

Wolter (5) successfully cultivated callus tissue from diploid quaking aspen on a chemically-defined medium. The medium consisted essentially of twice the concentration of salts used by Mathes in Medium 23, but replaces the coconut milk with 50 mg./l. inositol, and utilizes as auxin either 0.5 mg./l. 2,4-D alone or a mixture of 0.04 mg./l. 2,4-D and 1 mg./l. kinetin.

On January 26, 1965, both of Wolter's media were made up, and designated as W(A) for 2,4-D alone or W(B) for 2,4-D and kinetin. Another medium, W(C), was also made, substituting 0.5 mg./l. NAA and 3 mg./l. glycine (both used in Medium 23) for the auxins used by Wolter. Fe-EDTA was used in all three media, which were tested against the control Medium 23 in both agar and liquid cultures.

(1) Agar Cultures. For each of the four treatments, W(A), W(B), W(C), and 23, four pieces of triploid quaking aspen tissue (20-60 mg. each) were placed on five plates. Each piece was individually weighed at the beginning and end of the study, and was identified by number on the bottom of the plate. After

autoclaving, each medium was adjusted to pH 5.2-5.7. Plates were incubated for 28 days in the dark at 26°C.

The summary given in Table VII shows the average increase in fresh-weight growth of 34 mg. per piece for W(A), 14 mg. for W(B), 20 mg. for W(C), and 454 mg. for 23. The first three values represent 7.5, 3.1, 4.4%, respectively, of the growth obtained on Medium 23. There were no significant differences between replications and no interaction (Table VIII). Individual t-tests showed no differences between the three test media, but the growth on either W(A), W(B), or W(C) was far below that on Medium 23. Thus, these results show that growth on Wolter's media was unsatisfactory for the triploid form of quaking aspen.

TABLE VII

WEIGHT IN MG. OF THE INCREASE IN FRESH WEIGHT OF FOUR PIECES OF TRIPLOID QUAKING ASPEN TISSUE PER PLATE (REPLICATION)

Medium	Replications 1	2	3	4	5	Sum	Average Increase in Weight Per Piece, mg.	Per Cent Growth of Medium 23
W(A)	144	147	174	158	60	683	34	7.5
W(B)	97	51	46	57	38	289	14	3.1
W(C)	128	130	57	28	67	410	20	4.4
23	2055	1755	1631	1995	1635	9071	454	100
Sum	2424	2083	1908	2238	1800	10453		

TABLE VIII

TWO-WAY ANALYSIS OF VARIANCE OF DATA SUMMARIZED IN TABLE VII

SV	DF	SS	MS	F		
Treatments	3	2794	931.0	T/EE	1330	**
Replications	4	26	6.5	R/SE	1.6	NS
Experimental Error (TxR)	12	8	0.7	E/EE	< 1	NS
Sampling Error	60	250	4.2	($s_{\bar{x}}$ = 14.5)		
Total	79	3078				

(2) Liquid Cultures. At the same time that agar cultures were prepared, 60 ml. of Media W(A), W(B), W(C), and 23 were placed in each of five 250 ml. Erlenmeyer flasks, with no agar added. Into each flask was placed ten pieces of triploid quaking aspen tissue, weighing about 18 mg. each, which had been repeatedly subcultured in liquid Medium 23 made with dialyzed coconut milk. Flasks were placed on a shaker in the dark.

After 8 weeks' growth, the tissue in each flask was weighed and the data analyzed. The average increase in fresh weight of tissue in W(A) was 8 mg. per flask, 20 mg. for W(B), and 4 mg. for W(C). These weights were not significantly different from each other. However, growth in all three test media was significantly less than the average of 340 mg. per flask for tissue grown in Medium 23.

Thus, compared to Medium 23, the growth of triploid quaking aspen tissue is insignificant both on agar and in liquid cultures of Wolter's two media, as well as the hybrid Medium W(C). Preliminary observations of a similar test of agar Media W(A), W(B), and W(C), begun in July, 1965, tend to confirm these first results.

(3) Culture of Populus Species. The growth of five species of Populus, in addition to quaking aspen, was also tested on Wolter's media. Three pieces of tissue were placed on four plates each of W(A), W(B), W(C), and 23.

Subjective observations indicated that P. davidiana and P. alba can grow well on W(A), compared to growth on Medium 23, and fairly well on both B and C. P. grandidentata grew fairly well on A, but poor on B and C. P. canescens grew fair on C but poorly on A and B, and P. deltoides grew poorly on all three defined media.

Myo-inositol

After the apparent failure of Wolter's media to sustain the growth of triploid quaking aspen tissue, further tests were designed to determine whether myo-inositol can replace coconut milk in a defined medium. Several media (Table IX) were made up in both agar and liquid forms.

(1) Agar Cultures. Four pieces of triploid quaking aspen tissue (about 10 mg. each) were placed on each of three plates per medium. Plates were placed in the incubator April 2, 1965, and left in the dark at 26°C. for 24 days.

The increase in fresh weight per plate for each medium is given in Table X. In the defined Medium 32, the coconut milk of Medium 23 had been replaced with inositol, and the Fe-citrate replaced with Fe-EDTA. Also, twice the salts of Medium 23 were used. The weight increase of tissue grown on Medium 32 was 96% of that obtained on Medium 23, but growth was not as white nor as uniform. Medium 33, with all vitamins added, had fair growth but extensive necrosis. Media 30 and 31, which had different levels of salts with inositol and Fe-citrate added, had very poor growth. The results of this first trial indicate that inositol can replace coconut milk in the presence of Fe-EDTA, but

TABLE IX

THE COMPONENTS OF MEDIUM 23, AS WELL AS FOUR DEFINED MEDIA ARE GIVEN AS MG./L. IN THE FINAL SOLUTION

Chemical	23	30	31	32	33
Na_2SO_4	200	200	400	400	400
$MgSO_4 \cdot 7H_2O$	360	360	720	720	720
KNO_3	80	80	160	160	160
$Ca(NO_3)_2 \cdot 4H_2O$	200	200	400	400	400
KCl	66	66	142	142	142
$NaH_2PO_4 \cdot H_2O$	16	16	32	32	32
Fe-citrate	10	10	20	--	--
Fe-EDTA	--	--	--	5	5
H_2SO_4	50	50	50	50	50
$MnSO_4$	3	3	3	3	3
$ZnSO_4$	0.5	0.5	0.5	0.5	0.5
H_3BO_3	0.5	0.5	0.5	0.5	0.5
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025	0.025
$Na_2MoO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025	0.025
NAA	0.5	0.5	0.5	0.5	0.5
Glycine	3	3	3	3	3
Thiamine · HCl	0.1	0.1	0.1	0.1	0.1
Vitamins	--	--	--	--	--[a]
Myo-inositol	--	100	100	100	100
Coconut milk	100,000	100,000	100,000	100,000	100,000
Sucrose	20,000	20,000	20,000	20,000	20,000
Agar	8,000	8,000	8,000	8,000	8,000

[a]See text for names and amounts of vitamins.

not with Fe-citrate. Also, some vitamins appear to be inhibitory. The vitamins used included 0.4 mg./l. each of riboflavin and biotin, 0.1 mg./l. calcium pantothenate, 1 mg./l. each of thiamine and pyridoxine, and 4 mg./l. nicotinic acid. Vitamins had been frozen together in one solution for at least one year prior to use.

TABLE X

TOTAL INCREASE IN WEIGHT IN MG. OF FOUR PIECES OF TRIPLOID QUAKING ASPEN TISSUE PER PLATE, AFTER 24 DAYS' GROWTH AGAR CULTURES

Medium	Plate	Weight Increase Per Plate, mg.	Per Cent Growth of Medium 23
23	1	1105	100
	2	843	
	3	997	
30	4	113	
	5	72	9
	6	75	
31	7	79	
	8	93	7
	9	41	
32	10	942	
	11	998	96
	12	880	
33	13	544	
	14	527	55
	15	547	

(2) Liquid Cultures. Agar was omitted from part of each medium shown in Table IX. Fifty ml. of each medium were placed in each of three 250 ml. Erlenmeyer flasks. Ten pieces of triploid quaking aspen tissue (about 40 mg. apiece) were placed in each flask. This tissue had previously been grown in liquid Medium 23 made with dialyzed coconut milk. The flasks were then left on a shaker in the dark for 34 days.

Within a few days, pieces in all flasks began to grow as translucent yellow tissue. However, due to contamination, results were obtained for only two flasks per treatment. The total increase in fresh weight per flask, given in Table XI, shows that the best growth was obtained in Medium 32, as was also found in the agar cultures. In the liquid cultures, however, yields for all defined media were not significantly different from Medium 23. Growth in Media 31 and 32 was slightly better, and in Media 30 and 33, slightly less than in Medium 23. The results of this test indicate that in liquid cultures, inositol apparently can replace coconut milk in the presence of both Fe-EDTA and Fe-citrate.

TABLE XI

INCREASE IN FRESH WEIGHT PER FLASK IN MG. FOR TRIPLOID QUAKING ASPEN TISSUE PREVIOUSLY GROWN IN LIQUID MEDIUM 23D LIQUID CULTURES

Medium	Flasks			Color of Tissue
	1	2	Total	
23	3943	3205	7148	yellow
30	3185	3710	6895	brown
31	3840	4108	7948	yellow
32	3996	4104	8100	light yellow
33	3889	1844	5773	brownish-yellow

(3) Liquid Cultures: First Transfer. One half liter of each of the liquid media 23, 30, 31, 32, and 33 were placed in each of two 3-liter Erlenmeyer flasks. Into each pair of flasks, cut tissue was placed which had been previously grown in the same medium. All flasks were left on the shaker in the dark for 14 days.

All flasks became contaminated except those containing Medium 32. The tissue in these flasks was composed of yellow balls 1/8 to 3/8 inch in diameter, and weighed almost 17 g. in one flask and 12 g. in the other. Some of this tissue was used for the liquid cultures described later as part of the vitamin studies. The rest of the tissue was placed in two 3-liter flasks in liquid Medium 32. After two or three weeks, tissue placed in 32 became necrotic and was discarded.

Another, more extensive test, conducted by Mr. John Carlson of the Biochemistry Group, at the Institute, gave similar results. Tissue first grown in Medium 23D (dialyzed coconut milk), then subcultured and transferred to Medium 32 (CM replaced by inositol), produced excellent light yellow tissue. However, when this same tissue was again subcultured and transferred to fresh liquid 32, necrosis and cessation of growth occurred.

Clearly, the first transfer in Medium 32 was enhanced by something carried over in the tissue from the coconut milk, but which was exhausted during the second transfer.

(4) Species' Growth. The growth of tissue isolated from the five species of Populus, in addition to triploid quaking aspen, was also tested on the agar media listed in Table IX. On March 11, 1965, three clonal pieces of tissue from each species (about 5 mg. each) were placed on Media 23, 23E, 30, 31, 32, and 33.

After 28 days in the dark, pieces were weighed and a subjective analysis made of tissue quality. Populus deltoides grew very well on 33; P. canescens and P. davidiana grew poorly to fair on all defined media; P. alba could grow only poorly on 30; and both P. grandidentata and P. tremuloides produced fair tissue containing brown areas on 32. These tissues were not transferred to fresh medium because of their generally poor quality.

The results of these tests show that apparently myo-inositol alone cannot replace coconut milk in Medium 23, especially after the first transfer.

Vitamin Studies

Eleven chemically-defined media (Table XII) were prepared, in addition to Media 23 (Fe-citrate) and 23E (Fe-EDTA). Media 35-43 were made by adding one new vitamin to 32 for each new medium. Medium 34 received two vitamins; and 44, which received all new vitamins, was only used in the second transfer of liquid cultures. Recently isolated triploid quaking aspen tissue was tested on all media, which were prepared in both agar and liquid forms.

(1) Agar Cultures. On April 27, 1965, four pieces of tissue were placed on each of three plates per medium. The average initial weight of tissue per piece was 37 mg. in Replicate 1, 54 mg. for Replicate 2, and 41 mg. for Replicate 3. These estimates are based on four tissue samples weighed per replication. Plates were incubated 28 days in the dark at 24°C. One third of the original 156 plates of this test were lost from exceptionally heavy fungus contamination in the incubator – a condition subsequently corrected. However, at least one plate per medium remained uncontaminated. The analysis of variance shows that significant differences were present in the fresh weight increase of tissue grown on different media. Duncan's Multiple Range Test (7), adjusted for unequal subclass numbers, gave the results shown in Table XIII.

TABLE XII

MG./L. OF VITAMINS ADDED TO MEDIUM 32

Vitamin	Medium 34	35	36	37	38	39	40	41	42	43	44[a]
Nicotinic acid	0.5	0.5									0.5
Pyridoxine	0.1		0.1								0.1
Choline chloride				1.0							1.0
Hypoxanthine					2.5						2.5
Ca-pantothenate						0.1					0.1
Ascorbic acid							0.1				0.1
Riboflavin								0.1			0.1
Biotin									0.01		0.01
Sorbitol										1.0	1.0

[a]Medium 44 was used only in the second transfer of tissue in liquid culture.

Note: See Table IX for the composition of 32.

Medium 36 (32 plus pyridoxine) gave 75% of the growth obtained on 23E, but still had 56% more growth than on 23. Media 34 (nicotinic acid and pyridoxine) and 38 (hypoxanthine) did well; but 42 (biotin), 39 (calcium pantothenate), and 37 (choline chloride) were only fair. The rest gave generally poor results. Since tissue on all defined media were highly variable in size and had large necrotic areas, this tissue was not subdivided and transferred.

(2) Liquid Cultures: First Transfer. Tissue, described under (3) Liquid Cultures: First Transfer of the Myo-inositol study (p. 36), was also used in this experiment. Sixty ml. of liquid media having vitamin additions (Table XII) were placed in each of three 250 ml. Erlenmeyer flasks. Two controls of Media 23 and 23E brought the number of treatments to 13. Ten to twenty pieces

TABLE XIII

AGAR MEDIA, RANKED IN DESCENDING ORDER ACCORDING TO AVERAGE FRESH WEIGHT INCREASE IN MG. OF ASPEN TISSUE

(Significance was determined by Duncan's Multiple Range Test corrected for unequal subclass numbers)

Rank	Medium	Vitamins Added to Medium 32	No. plates Analyzed	Av. Increase Per Plate, mg.	Significance	Per Cent Increase
1	23E	CM	2	818		4316
2	36	Pyridoxine	2	616		2851
3	23	CM	1	459		3122
4	34	Nicotinic acid, pyridoxine	3	407		2320
5	38	Hypoxanthine	1	379		1770
6	42	Biotin	6	283		1613
7	39	Ca-Pantothenate	2	262		1452
8	37	Choline chloride	1	201		939
9	32	(Inositol, thiamine)	2	194		1078
10	35	Nicotinic acid	1	193		1316
11	40	Ascorbic acid	3	179		1022
12	43	Sorbitol	2	159		839
13	41	Riboflavin	3	139		793

of cut tissue were placed in each flask in estimated weights of 455, 462, and 392 mg. per flask, respectively, for Replicates 1, 2, and 3 of each treatment. All flasks were left on the rotary shaker in the dark for 4 weeks.

Table XIV gives the performance of each medium ranked by descending net increase in weight. The per cent increase gives almost the same order of ranking. The analysis of variance showed highly significant differences between media. However, Duncan's Multiple Range Test, as well as selected t-tests, indicated no significant differences. Another analysis of variance, excluding data from Medium 23, showed no differences between the remaining media. Apparently the only significant variation can be attributed to tissue grown on Medium 23; which had, in this case, much better growth than tissue on 23E. This is just the reverse of the results of the previous test (Table XIII).

TABLE XIV

LIQUID CULTURES: FIRST TRANSFER – RANKED IN DESCENDING ORDER ACCORDING TO THE INCREASE OF FRESH WEIGHT IN MG. OF TRIPLOID QUAKING ASPEN TISSUE

Rank	Medium	Vitamins Added to Medium 32	Average Increase Per Flask Net	Per Cent
1	23	CM, Fe-citrate	5088	1066
2	42	Biotin	3951	806
3	43	Sorbitol	3510	665
4	23E	CM, Fe-EDTA	3111	579
5	40	Ascorbic acid	2964	579
6	37	Choline chloride	2839	519
7	32	(Inositol, thiamine)	2780	537
8	41	Riboflavin	2525	491
9	38	Hypoxanthine	2287	424
10	36	Nicotine-pyridoxine	1666	290
11	34	Nicotinic acid	1627	273
12	39	Ca-pantothenate	1202	176
13	35	Pyridoxine	1027	124

(3) Liquid Culture: Second Transfer. On May 25, 1965, three sets of ten pieces each were cut from tissue grown in each defined liquid media described under (2) of this study. Each set of tissue was weighed (1000-2000 mg.) and placed in a 250 ml. flask. After 4 weeks on the rotary shaker, tissue was again weighed, then discarded.

Table XV ranks the media in descending order according to the fresh weight increase of tissue. Per cent increases also are included. The data of Table XV were combined with that in Table XIV, resulting in the bar graphs of Fig. 11 and 12. The increase in weight for two successive transfers is shown in Fig. 11, and the per cent increase of the same tissue is given in Fig. 12. There is a very close relationship between the rankings of media by weight increase and per cent increase. However, the differences in growth between the first and second transfer, for the same medium, vary considerably whether plotted according to net weight increase or per cent increase. Generally, growth for several media was good during the first transfer, but rather poor in the second. The exception to this was with Medium 32 plus calcium pantothenate (Medium 39).

Based on these results, studies are now being planned to test various combinations of sets of three vitamins. Another study will start with all vitamins, including vitamin B_{12}, and successive media will have one vitamin at a time eliminated. These tests will run through three transfers.

TABLE XV

LIQUID CULTURES: SECOND TRANSFER – DEFINED MEDIA RANKED IN DECREASING ORDER ACCORDING TO THE AVERAGE INCREASE IN WEIGHT PER FLASK, IN MG. OF TRIPLOID QUAKING ASPEN TISSUE

Rank	Medium[a]	No. Flasks	Weight Increase Per Flask, mg.	Per Cent Increase
1	23	3	2253	1141
2	23D	2	1168	859
3	39	2	975	811
4	32	2	691	458
5	40	3	507	472
6	35	3	348	212
7	38	3	332	271
8	37	3	271	228
9	43	1	265	172
10	36	2	186	169
11	34	2	148	95
12	42	1	116	91
13	44	1	91	41

[a]All flasks of Media 23E and 41 were contaminated.

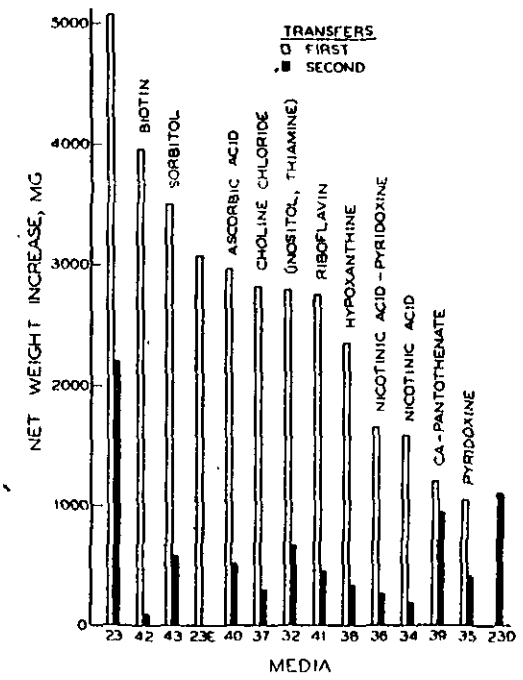

Figure 11. Bar Graph of the Increase in Fresh Weight of Quaking Aspen Tissue, Grown in Liquid Defined Media Through Two Successive Transfers. Media 23, 23E, and 23D Were Controls Made With Coconut Milk. Each Defined Medium Was Made by Adding the Listed Vitamin to Medium 32

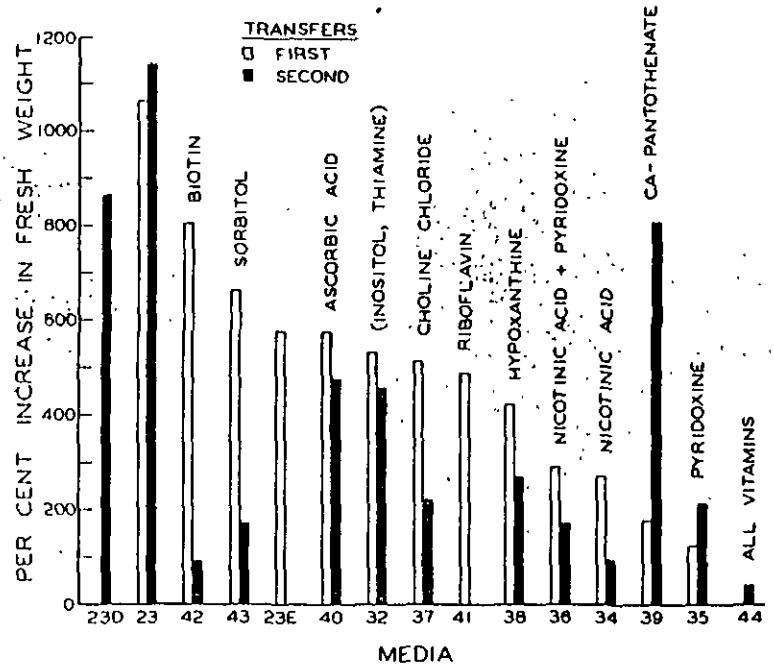

Figure 12. Bar Graph of the Per Cent Increase in Fresh Weight of Quaking Aspen Tissue, Grown in Liquid Media Through Two Successive Transfers

OTHER STUDIES

In addition to studies of variation and nutrition, a few exploratory tests were run on pollen and single-cell cultures. Several strains of triploid aspen tissue were recovered and propagated, and one piece of callus tissue was found which had several roots and two elongating shoots. These events are recorded here as general interest, but will be pursued in greater depth at later stages of this program.

POLLEN AND SINGLE-CELL CULTURES

On January 27, 1965, male-catkin bearing branches from one diploid quaking aspen tree (T-10-60) were collected and forced in the greenhouse. Four days later, microspores were present when catkins were 2.3 cm. long and just starting to elongate. Anthers were red at this stage. Under sterile conditions, anthers were squashed in water and the mixture spread on the surface of agar Medium 23. After two days' incubation, microspores began enlarging and showed signs of a budding type of proliferation.

Freshly shed pollen was also dusted onto agar February 4, 1965, as well as being streaked and poured on Media 23, W(A), W(B), W(C), and 23 plus 0.5 mg./l. kinetin. However, after four days most material was contaminated.

Two plates each were maintained from the microspore and pollen grain inoculations. On March 15, colonies of cells from each plate were transferred to both liquid and agar Medium 23. After one month, very few colonies of cells were found on agar, and single cells were not abundant in the liquid cultures. All material was discarded. These results show some indication, however, that both pollen colonies on agar and single-cell suspensions may be obtained under optimum environmental conditions.

TISSUE STRAINS

Normal pieces of callus tissue from triploid quaking aspen are yellow-brown with white, rapidly dividing tissue on the top surface (Fig. 4). Several variants of this tissue are listed below. Samples of most strains are now embedded and will be sectioned for examination of internal structure.

Root-Nodule Tissue

On October 20, 1964, a large piece of tissue was found among the cultures which had three small shoots and several white swellings on its roots. This was transferred to Medium 23 plus 10 mg./l. gibberellic acid (GA), and placed under strong fluorescent-incandescent light. By November 11, reddish nodules, 2.0 to 2.7 mm. in diameter, were evident on the roots (Fig. 13).

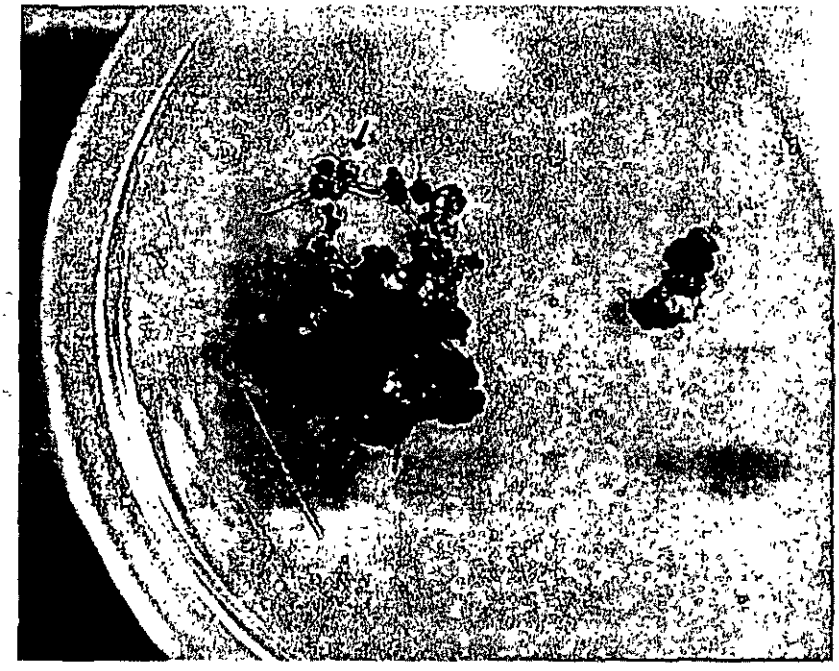

Figure 13. Red Nodules, 2.0 to 2.7 mm. in Diameter, on Roots of Triploid Quaking Aspen Tissue Grown on 23 + GA Under Light.

November 17, two nodules were removed and dissected, and one half of each placed in FPA and Craf III fixatives. Two other nodules were dissected and one half each placed on Media 23 and 23 plus GA. For the first couple of weeks the tissue on Medium 23 plus GA grew faster than that on 23. But by December 16, the growth of both was about equal and pieces were about 1/2 cm. in diameter. After 63 days' growth (January 18), each piece was about 1.5 cm. in diameter, and slightly better growth was evident on Medium 23 (Fig. 14 and 15). At this time, white tissue from each piece was subdivided and placed on both Media 23 and 23 plus GA. The best growth was on 23. As of July 1, these tissues had been subcultured six times and are now being maintained as three excellent, white and uniform strains: (1) 3x-Na (GA on 23E), (2) 3x-Nb (23 on 23), and (3) 3x-Nc (GA on 23).

In order to test whether nodules can be induced, a large piece of rooted P. canescens tissue was placed on Medium 32 plus GA under light. No swellings were evident on the heavy roots. After one week, nodulelike swellings appeared, which grew to about 1/2 cm. in diameter. Unfortunately, this tissue became infected and was discarded.

Friable Strain

During the growth of tissue in closed bottles (Fig. 8), pieces of triploid quaking aspen tissue occasionally were found which were straw colored and friable (crumbly). This tissue was isolated from several pieces in clonal lines. As of July 1, the most uniform strain (3x-f5) had been subcultured three times (Fig. 16). The other lines were discarded. The strain 3x-f5 is light-sensitive and turns purple in the presence of continuous light. However, one piece is currently being cultured which began to grow white tissue while in the light.

Figure 14. Root-Nodule Tissue Grown on Medium 23 for 63 Days

Figure 15. Root-Nodule Tissue Grown on 23 + GA for 63 Days

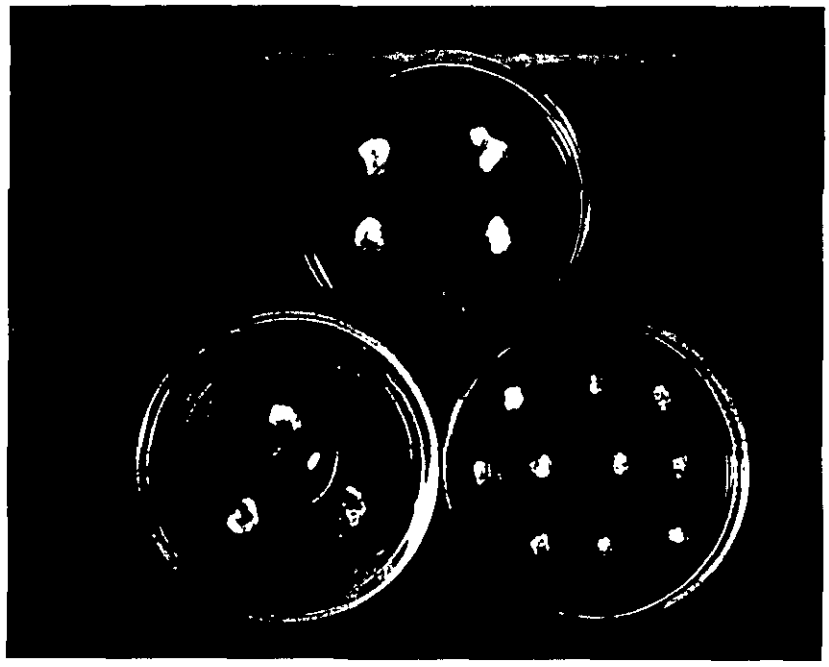

Figure 16. Three Friable Strains of Triploid Quaking Aspen Tissue. Top: Liquid-Grown Tissue on Solid 23. Bottom Left: 3x-F1. Bottom Right: 3x-f5

Light-Resistant Strain

Also, during the closed bottle test, three pieces of triploid quaking aspen tissue were individually placed on Medium 23 slants in closed bottles. Bottles were left on top of the bench, receiving only the normal fluorescent lighting in the lab. The pieces turned red, but also developed large patches of dense white tissue on the top surfaces. Subcultures of the white tissue continue to give white tissue, and the lower part of each callus is light green instead of red.

Liquid-Grown Strain

Triploid quaking aspen tissue grown in liquid 23D, then placed on agar 23 produces a soft, friable, honey-colored tissue (Fig. 16). This tissue turns

brown if crowded on the plate, but is being maintained as a friable strain for possible single-cell cultures.

DIFFERENTIATED TISSUE

In November, 1964, one piece of triploid quaking aspen tissue was found which had been growing on Medium 23 plus 0.5 mg./l. kinetin. This tissue had three 1-cm.-long roots and two elongating shoots about 1.2 cm. in height (Fig. 17). After three transfers of two weeks each, the tissue became contaminated and was killed and fixed. It is now embedded in paraffin, awaiting sectioning and examination of its internal anatomy and morphology. The large scale production of differentiated tissue, such as this piece, is the long range goal of this entire program. Presumably, each differentiated piece would be capable of growing into a mature aspen tree. Such a population of genetically uniform trees would then be the basis of a sophisticated breeding and selection program.

Figure 17. Triploid Quaking Aspen Tissue Having Three Roots and Two Elongating Shoots. Cover Glass is One Inch Square

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